Mj. Int. J. Sci. Tech. **2008**, *2*(02), 418-430

Maejo International

Journal of Science and Technology

ISSN 1905-7873

Available online at www.mijst.mju.ac.th

Full Paper

On-line preconcentration and determination of tetracycline residues in milk using solid-phase extraction in conjunction with flow injection spectrophotometry

Prinya Masawat [1,*], Sutthinee Mekprayoon [1], Saisunee Liawruangrath [2], Suphachock Upalee [2], and Napaporn Youngvises[3]

[1] Department of Chemistry, Faculty of Science, Naresuan University 65000, Thailand

[2] Department of Chemistry, Faculty of Science, Chiang Mai University 50200, Thailand

[3] Department of Chemistry, Faculty of Science and Technology, Thammasat University, 12121, Thailand

* Corresponding author, e-mail : prinyam@nu.ac.th

Received: 9 January 2008 / Accepted: 20 July 2008 / Published: 21 July 2008

Abstract: A simple, cheap and highly sensitive system with on-line preconcentration using solid-phase extraction in conjunction with flow injection spectrophotometry for the determination of tetracycline residues in milk samples is described. C_{18} was used as packing material in a designed minicolumn used for preconcentration of tetracyclines. Tetracycline standard or sample solutions were dissolved in a mixed buffer solution of pH 4.0 containing boric acid, citric acid and sodium phosphate, then loaded to the minicolumn for 6 min followed by elution with a solution containing methanol : mixed buffer solution (40:60 by volume) of pH 6.5 The absorbance of the eluate was measured at 370 nm. The calibration graph was linear in the range of 0.20-1.00, 0.20-4.00, and 0.20-1.00 mg L^{-1} for tetracycline (TC), oxytetracycline (OTC), and chlortetracycline (CTC) respectively. The limits of detection were 0.08, 0.10, and 0.09mg L^{-1} for TC, OTC, and CTC respectively. Relative standard deviations for 20 replicated determinations of 0.20, 0.40, and 0.60 mg L^{-1} of TC were 7.03, 7.23, and 6.55 % respectively. Per cent recoveries for four commercial types of milk: U.H.T., pasteurised, raw, and sterilised milk were in the range of 86–109 (TC), 90–109 (OTC), and 89–108 (CTC). The sample throughput was 6 h^{-1}.

Keywords: tetracycline, oxytetracycline, chlortetracycline, tetracycline residues, solid-phase extraction flow injection spectrophotometry

Mj. Int. J. Sci. Tech. **2008**, *2*(02), 418-430

Introduction

The tetracyclines (Figure 1) have served for decades as an important class of antibiotics for the health of food-producing animals. They are antibiotics with a broad antibacterial spectrum and bacteriostatic activity, and have a good activity against acute diseases caused by Gram-positive and Gram-negative bacteria. They are licensed for use in a variety of food-producing animals including cattle, pigs, sheep, poultry and fish [1]. The use of these drugs has become a serious problem as regards their residues in milk or meat, which can be directly toxic to or else cause allergic reactions in some hypersensitive individuals. Even more important, low-level doses of the antibiotic in foodstuffs consumed for long periods can lead to problems regarding the spread of drug-resistant microorganisms. To ensure human food safety, maximum residue limits (MRLs) have been set for tetracycline, chlortetracycline and oxytetracycline in a number of tissue types, e.g. 0.3 mg kg^{-1} in liver, 0.6 mg kg^{-1} in kidney, 0.2 mg kg^{-1} in eggs and 0.1 mg kg^{-1} in milk and muscle tissues [2-3].

Generally, the presence of tetracyclines in milk arises from their use as part of therapy to treat animal diseases such as bovine mastitis and sometimes, in low concentrations, as constituents of animal feed to increase feed utility in accelerating animal growth. The availability of a simple and automatic method to control traces of these antibiotics in milk is of great analytical interest. High-performance liquid chromatography (HPLC) is one of the most popular and sensitive techniques for this purpose. Firstly, however, isolation of the tetracyclines from milk using some type of extraction and/or clean-up is required. One widely-used technique is solid-phase extraction (SPE). The tetracyclines are extracted then subjected to clean-up on a C_8 [4], a C_{18} [5] and a copolymeric [6] SPE column followed by injection in the HPLC column. Because almost without exception these methods are time-consuming, the availability of alternative methods is always desirable.

Flow injection analysis (FIA) is a well-known technique that offers improvement in most batch methods, especially in the high sample throughput. For the tetracyclines, there are a few FIA methods available with several types of detection such as chemiluminescence [7], electrochemical method [8-9], and spectrophotometry [10-11]. The spectrophotometric FIA detection [10] for the tetracyclines is based on the formation of a coloured product by their reaction with 4-aminophenazone and hexacyanoferrate(III). However, this method has a limited concentration range. Under optimised conditions, the tetracyclines were determined in the range of 1-20 and 20-250 mg L^{-1}. The lowest limit of detection is 0.2 mg L^{-1} for doxycycline [10].

SPE off-line performance is both time consuming and complicated, and thus it is usually the slowest step of the analysis. One of the possibilities to facilitate and accelerate the analysis of samples is to integrate the mentioned preparation of samples with the use of SPE directly to FIA. There are some major trends in the on-line coupling of SPE to FI manifolds: (i) direct application of the real samples, without any pre-treatment, to the flow injection system ; (ii) integration of reaction (retention) and detection; and (iii) miniaturisation as a means of reducing sample and reagent consumption. In the present work, a simple system of on-line preconcentration using SPE in conjunction with flow injection spectrophotometry with low instrumental cost, high sensitivity and low detection limit has been developed for the determination of tetracycline residues in milk. The method is based on the retention of the tetracyclines onto C_{18} and elution with mixed buffer solution and methanol adjusted to pH 6.5. The optimum conditions of this system were also studied such as wavelength, size and length of the minicolumn, type of eluent, preconcentration time, and flow rate of reagent. The performance of the developed system provided an enrichment factor [12] of about 30.

Mj. Int. J. Sci. Tech. **2008**, *2*(02), 418-430

Materials and Methods

Chemicals and reagents

All chemicals were of analytical-reagent grade, except where otherwise stated. Deionised water was used.

Tetracycline (TC) standard solution (SIGMA, U.S.A) was dissolved in acetate buffer (pH 4.0). Working standard solutions (0.1-1.0 mg L^{-1}) were daily prepared by adequate dilution of 50 mg L^{-1} TC, the stock standard solution, in the acetate buffer.

Mixed buffer solutions of pH ranging from 2.0 to 7.5 were prepared by dissolving boric acid (Merck, Germany) (12.37 g), citric acid (Fisher Scientific, UK) (10.51 g) and trisodium phosphate dodecahydrate (Fisher Scientific, UK) (38.01 g) in de-ionised water, then diluting to 1 L.

A mixture of methanol (LAB-SCAN, Ireland) and mixed buffer solution (pH 6.5) at 40:60 volume ratio was used as eluent.

The reagents used for extraction of tetracycline antibiotics from milk were prepared from trichloroacetic acid (Fluka, Switzerland), citric acid monohydrate (Merck, Germany), disodium hydrogenphosphate dihydrate (Merck,Germany), and ethylenediaminetetraacetic acid (EDTA) disodium salt (Fisher Scientific, UK).

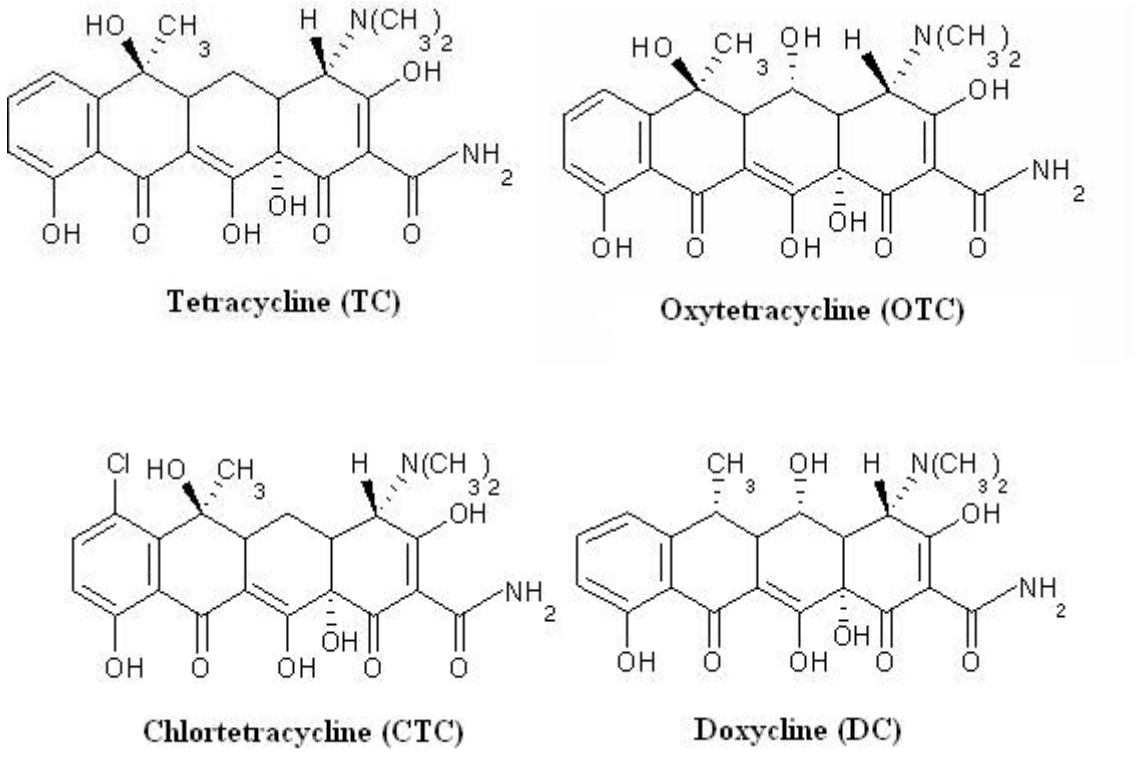

Figure 1. Structures of tetracycline antibiotics

Sample preparation procedures

Tetracycline residues were determined in four commercial types of milk: U.H.T., pasteurised, sterilised, and raw milk obtained from a local market of Phitsanulok area in 2005. All samples were

Mj. Int. J. Sci. Tech. **2008**, *2*(02), 418-430

collected in labeled dark plastic bags, transported to the laboratory and stored for a short time at 4°C prior to analysis.

An 5 mL aliquot of milk was placed in a glass centrifuge tube and 5 mL of 1.0 M trichloroacetic acid was added with shaking. Then 15 mL of Mcllvain buffer (consisting of 10.5 g citric acid monohydrate, 14.2 g disodium hydrogenphosphate dihydrate, and 30.25 g EDTA disodium salt in 1 L of de-ionised water) was added and the mixture was centrifuged at 6000 rpm for 15 min. The supernatant was then passed through a filter with 0.45-μm pore size followed by application to an online solid phase extraction-flow injection analysis (SPE-FIA) system.

The on-line SPE-FIA

The on-line SPE with flow injection spectrophotometric manifold is shown in Figure 2. A stream of solution (R1) containing a tetracycline (standard or sample) and eluent (R2) were controlled by a peristaltic pump (P, BIO RED, U.S.A). The tetracycline was loaded to a designed minicolumn (Figure 3) packed with C_{18} resin (Alltech, U.S.A). This minicolumn replaced the sample loop of the injection valve (Omnifit, England). The tetracycline adsorbed on the resin was desorbed by a stream of eluent (methanol : pH 6.5 buffer solution = 40:60) and carried past a flow-through cell (Hellma, Germany) located in the spectrophotometer (Ultrospec 4050, LKB BIOCHROM, UK) connected to a computer. All the tubing used was Teflon (0.8 mm id.), except the pump tubing which was Tygon. Connections were made with flexible T-piece sleeves . The absorbance of the tetracycline, which is proportional to its concentration, was recorded at 370 nm. Therefore, the amounts of the tetracycline in sample can be calculated from the calibration curve plotted between peak area and tetracycline concentration.

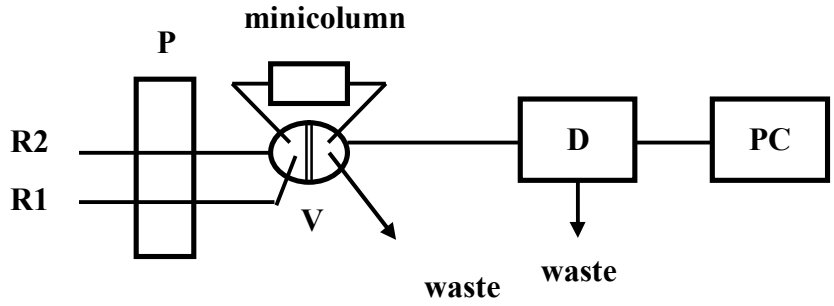

Figure 2. Flow injection manifold for the determination of tetracycline residues in milk: P = peristaltic pump, V = injection valve, D = detector (spectrophotometer), PC = personal computer, R_1 = sample, R_2 = eluent

Recovery experiments, quantitative evaluation and detection limits

Milk samples were fortified at 0.3, 0.5, 0.7, and 0.9 mg L^{-1} by adding a solution of the tetracycline. The per cent recoveries of four commercial milk samples were in the range of 86-109% (for TC), 90-109% (for OTC), and 89-108% (for CTC). The detection limits calculated by using a

Mj. Int. J. Sci. Tech. **2008**, *2*(02), 418-430

signal-to-noise (S/N) ratio of 3 [13] were 0.08, 0.10, and 0.09 mg L^{-1} for TC, OTC, and CTC respectively. The quantity of each of the tetracyclines was calculated by applying the standard addition method.

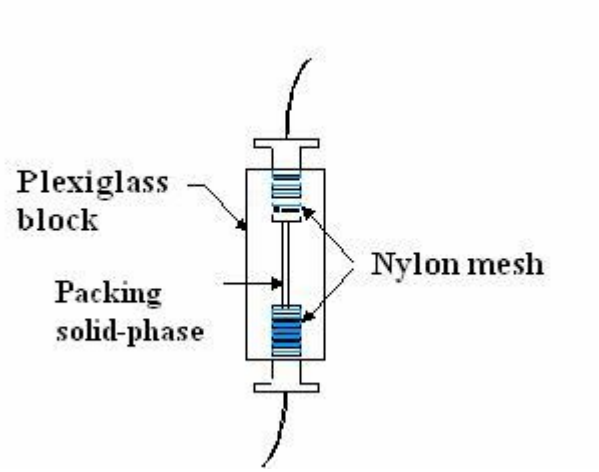

Figure 3. Minicolumn for the determination of tetracycline residues in milk (packing solid phase should be C_{18} resin, 35-75 μm)

Results and Discussion

In order to develop the method, various experiments were carried out. First, the absorption spectra were recorded to select the working wavelength. The influences of the following variables were then studied and the optimum values established: (a) preconcentration time; (b) pH of standard solutions; (c) type and pH of eluent; (d) flow rate; and (e) size and length of the designed minicolumn. Table 1 shows the initial conditions for optimisation of the on-line SPE-FIA using univariate method. After optimisation, the analytical figures of merit were studied. Finally, the developed procedure was applied to real milk samples.

Table 1. Initial conditions for optimisation of on-line SPE-FIA system for determination of tetracyclines

Condition	Initial value
wavelength	370 nm
TC concentration	0.1, 0.5 and 1.0 mg L^{-1}
eluent (methanol : pH 7.0 mixed buffer solution)	50: 50 by volume
Flow rate	0.5 mL min^{-1}
Size of minicolumn	1.0 cm in length, 0.3 cm i.d.
Solid-phase packing	C_{18}, 35-75 μm

Mj. Int. J. Sci. Tech. **2008**, *2*(02), 418-430

Absorption spectra

The UV-VIS spectrum of TC (5 mg L^{-1} in mixed buffer solution of pH 7.5) was obtained by batch method. The maximum absorption wavelength of TC was 370 nm as shown in Figure 4. This value was also obtained for OTC and CTC. Because of their polarity, the tetracyclines cannot be eluted from a polar normal-phase SPE cartridge (i.e. florisil, cyanoproply, silica) and reverse-phase SPE cartridges are more commonly used [14]. The C_{18} clean-up method combined with Mcllvaine buffer containing EDTA disodium salt for extraction was introduced in 1983 for the first time and appears to be the current standard method for the extraction and clean-up of tetracyclines in foods. Therefore, C_{18} was chosen as the packing material packed in the designed minicolumn, and the working wavelength was fixed at 370 nm.

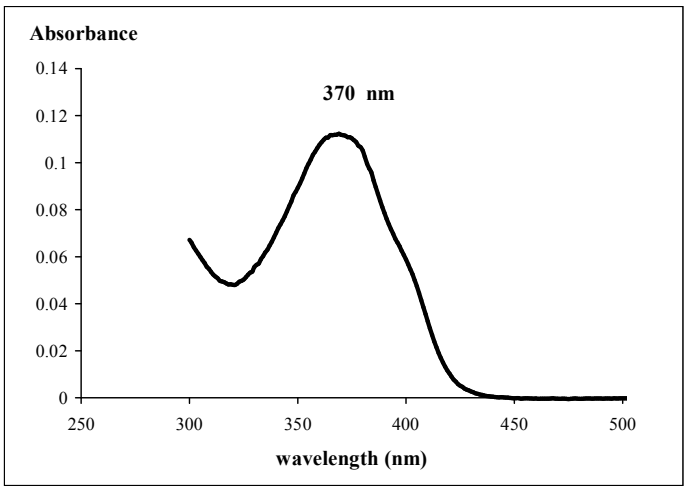

Figure 4. UV-VIS absorption spectrum of TC (5 mg L^{-1} in pH 7.5 mixed buffer solution)

Preconcentration time

The influence of preconcentration time on the analytical sensitivity was investigated between 2-8 min. As expected, the analytical sensitivity progressively increased with increasing preconcentration time (Figure 5). However, the peak shape of TC became distorted and broadened (not useable) when the preconcentration time was higher than 6 min. Thus, the preconcentration time of 6 min was chosen for the experiment.

pH of TC standard solution

The influence of pH of the mixed buffer solution used for dissolving the TC standard was examined by varying the pH in the range of 2.0-7.5. As shown in Figure 6, the optimum pH that gave a high sensitivity for the analytes was in the acidic range (pH 3-4). At pH values higher than 4, the analytical sensitivity decreased significantly. A working pH value of 4.0 was chosen since the analytical sensitivity was highest.

Mj. Int. J. Sci. Tech. **2008**, *2*(02), 418-430

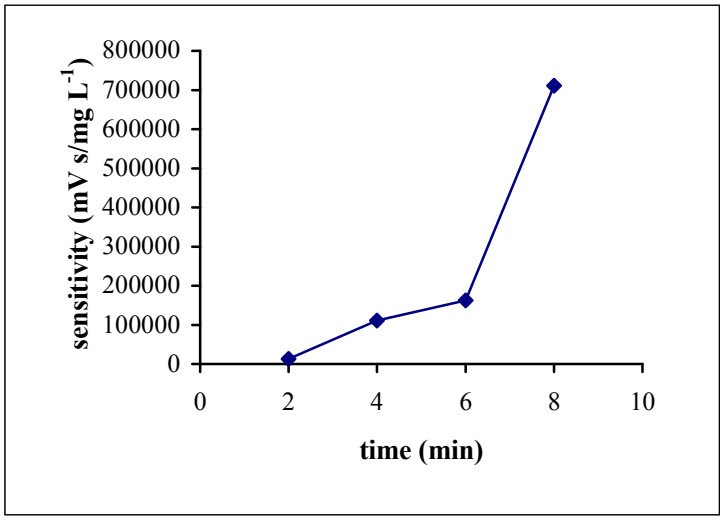

Figure 5. Effect of preconcentration time on analytical sensitivity for tetracycline standard solution

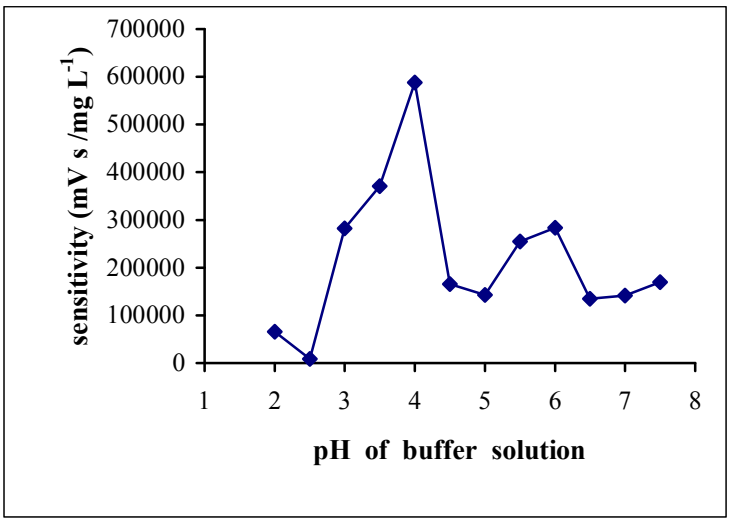

Figure 6. Effect of pH of buffer solution (for dissolving TC) on analytical sensitivity for tetracycline standard solution

Type and pH of eluent

The eluents containing methanol:mixed buffer solution at different ratios (10:90, 20:80, 30:70, 40:60, 50:50, and 60:40) were tested. It was found that bubbles were formed when more than 50% of methanol was used and the peak shapes became broadened when more than 60%of the buffer solution was used. The ratio of 40:60 for methanol to buffer solution was therefore chosen as eluent for TC. The influence of pH of the buffer solution used as eluent was investigated by varying the pH in the range of 2.0-7.5. As shown in Figure 7, a working pH value of 6.5 was chosen since the sensitivity was highest at this pH.

Mj. Int. J. Sci. Tech. **2008**, *2*(02), 418-430

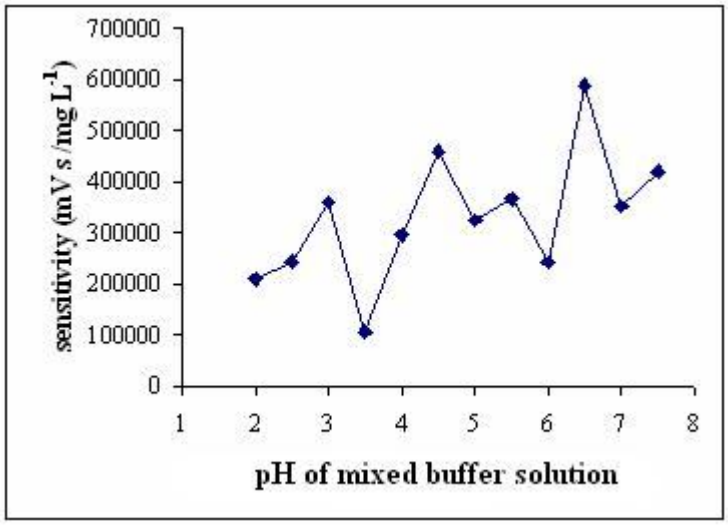

Figure 7. Effect of pH of mixed buffer solution (eluent) on analytical sensitivity for tetracycline standard solution

Flow rate

The effect of flow rate was investigated between 0.3-0.8 mL min^{-1}. When the flow rate increased the analytical sensitivity (Figure 8) and the elution time progressively decreased and, consequently, the sampling frequency increased (3 h^{-1} at 0.3 mL min^{-1} to 7 h^{-1} at 0.6 mL min^{-1}). At lower flow rate, the peaks of TC were broader than those obtained at higher flow rate. However, no peak of TC at low concentration was detected when using flow rate higher than 0.6 mL min^{-1}. This is apparently due to low sorption of TC on the C_{18} resin. As a compromise, a 0.5 mL min^{-1} flow rate was chosen.

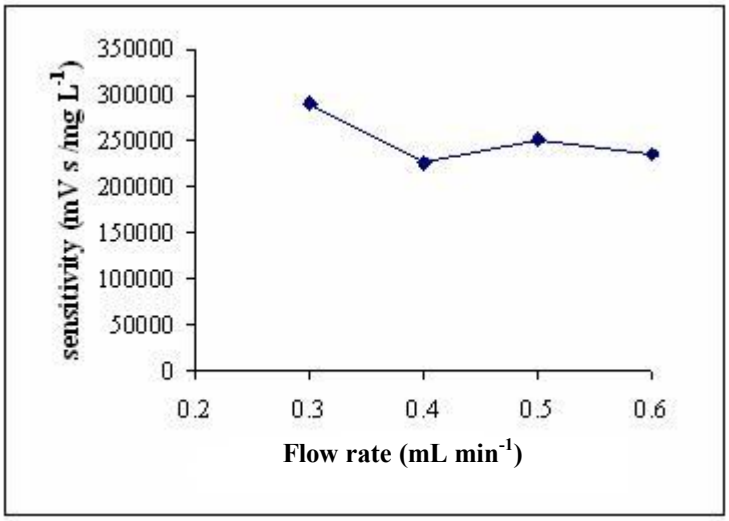

Figure 8. Effect of flow rate on analytical sensitivity for tetracycline standard solution

Mj. Int. J. Sci. Tech. **2008**, *2*(02), 418-430

Size and length of designed minicolumn

In order to determine tetracycline residues in milk, preconcentration on the C_{18} resin packed in a minicolumn is the crucial step. The influence of size and length of the designed minicolumn was then tested as exhibited in Table 2. It was found that the analytical sensitivity was strongly dependent on the length of the minicolumn. However, as the length increased the total analysis time also increased. Thus, the minicolumn type 1 (1.0 cm length and 0.3 cm i.d.) was chosen for the preconcentration purpose.

Table 2. Effect of minicolumn dimension on analytical sensitivity for tetracycline standard solution

Type	Size of minicolumn		Cell volume (cm^3)	Analytical sensitivity* (mV s /mg L^{-1})
	Length (cm)	i.d. of minicolumn (cm)		
1	1.0	0.3	0.071	374162
2	3.0	0.3	0.210	661810

* in triplicate

Figures of merit

Using the optimum conditions obtained (Table 3), figures of merit of the method proposed for the determination of tetracyclines are provided in Table 4. The linear range of the tetracyclines was 0.08-1.00 mg L^{-1} for TC, 0.20-4.00 mg L^{-1} for OTC and 0.20-1.00 mg L^{-1} for CTC, with regression coefficients higher than 0.99 in all cases. It can be seen from Table 4 that the detection limits of TC, OTC, and CTC are lower than the MRL of each tetracycline [2-3]. Thus, the proposed on-line SPE-FIA is suitable for the determination of the tetracyclines in food samples.

Table 3. Optimum values of the variables of on-line SPE with flow injection spectrophotometric system for the determination of tetracyclines

Variable	Range studied	Optimum value
1. Wavelength of tetracycline standard solution (nm)	300-700	370
2. Preconcentration time (min)	2-8	6
3. pH of tetracycline standard solution	2.0-7.5	4.0
4. pH of eluent	2.0-7.5	6.5
5. Ratio of eluent (methanol:buffer solution)	10:90, 20:80, 30:70, 40:60, 50:50, 60:40	40:60
6. Size of minicolumn	length (cm) i.d.(cm)	length (cm) i.d. (cm)
	1.0 0.3	1.0 0.3
	3.0 0.3	
7. Flow rate (mL min^{-1})	0.3-0.8	0.5

Mj. Int. J. Sci. Tech. **2008**, *2*(02), 418-430

Table 4. Figures of merit of the proposed method for determination of tetracyclines

Standard solutions	Linear range (mg L^{-1})	Detection limit (mg L^{-1}) [13]	Calibration curve	
			Sensitivity (mV s /mg L^{-1})	r^2
TC	0.08-1.00	0.08	321198	0.9947
OTC	0.20-4.00	0.10	171091	0.9909
CTC	0.20-1.00	0.09	162191	0.9916

Analysis of milk samples

The proposed method was applied to the determination of TC, OTC and CTC in four commercial types of milk: U.H.T., pasteurised, raw, and sterilised. For this purpose, different amounts of the tetracyclines were added to each sample in order to carry out the recovery study. It was found that, fortunately, tetracyclines were not detected in any of the original samples. From Table 5, the per cent recoveries obtained were in the range of 86–109 for TC, 90–109 for OTC, and 89–108 for CTC.

Unlike HPLC, the proposed method obviously cannot discriminate between TC, OTC and CTC in a sample. Nevertheless, it is quite convenient to operate, uses less reagent and produces less waste. Moreover, since the tetracyclines can be preconcentrated on the minicolumn by an enrichment factor of 30 [12], the method is good enough for analysis of milk samples.

Table 5. Percent recovery obtained from the proposed method for determination of tetracyclines in four types of milk

Milk sample	Standard	Added (mg L^{-1})	Found (mg L^{-1})	% Recovery (n = 3)
U.H.T.	TC	0.30	0.26	86
		0.50	0.54	109
		0.70	0.73	105
		0.90	0.86	96
	OTC	0.30	0.32	107
		0.70	0.64	92
		0.90	0.94	104
	CTC	0.30	0.32	106
		0.50	0.47	95
		0.90	0.91	101

Mj. Int. J. Sci. Tech. **2008**, *2*(02), 418-430

Milk sample	Standard	Added (mg L^{-1})	Found (mg L^{-1})	% Recovery (n = 3)
Pasteurised	TC	0.30 0.50 0.70 0.90	0.26 0.54 0.73 0.88	88 108 104 97
	OTC	0.30 0.50 0.70 0.90	0.27 0.54 0.70 0.88	90 109 101 98
	CTC	0.30 0.50 0.70 0.90	0.27 0.53 0.73 0.87	89 107 105 96
Raw	TC	0.30 0.50 0.70 0.90	0.28 0.51 0.74 0.87	94 101 105 97
	OTC	0.30 0.50 0.90	0.27 0.54 088	90 109 98
	CTC	0.30 0.50 0.70 0.90	0.32 0.48 0.66 0.93	108 97 95 103
Sterilised	TC	0.30 0.50 0.70 0.90	0.31 0.51 0.65 0.93	103 103 94 103
	OTC	0.30 0.50 0.70 0.90	0.30 0.49 0.69 0.90	101 100 100 100
	CTC	0.30 0.50 0.90	0.30 0.49 0.90	102 99 100

Mj. Int. J. Sci. Tech. **2008**, *2*(02), 418-430

Conclusions

The first use of on-line SPE-FIA for the determination of tetracycline antibiotics in real milk samples was described. It is highly sensitive as a result of the preconcentration of the tetracyclines onto the C_{18} resin. Only small amounts of sample, sorbent and reagent are required. A high enrichment factor can be attained by employing large sample volumes, making it possible to work at different concentration levels. Tetracycline residues, however, were not detected in the studied milk samples collected.

Acknowledgements

We gratefully acknowledge the Faculty of Science, Naresuan University for funding this research. Some of the devices and Windows applications were developed in cooperation with Alpha Flow Research, Chiang Mai University.

References

1. Y. Debuf (Ed.), "The Veterinary Formulary", Pharmaceutical Press, London, **1988**, p.97.
2. EC Regulation 2377/90, incorporating amending regulation 281/96.
3. Commission Regulation No. 508/99, *Off. J. Eur. Commun.,* No. L609, March **1999**.
4. N. Furusawa, "Isolation of tetracyclines in milk using a solid-phase extracting column and water eluent", *Talanta*, **2003**, *59*, 155-159.
5. A. L. Cinquina, F. Longo, G. Anastasi, L. Giannetti, and R. Cozzani, "Validation of a high-performance liquid chromatography method for the determination of oxytetracycline, tetracycline, chlortetracycline and doxycycline in bovine milk and muscle", *J. Chromatogr. A*, **2003**, *987*, 227-233.
6. W. C. Andersen, J. E. Roybal, S. A. Gonzales, S. B. Turnipseed, A. P. Pfenning, and L. R. Kuck, "Determination of tetracycline residues in shrimp and whole milk using liquid chromatography with ultraviolet detection and residue confirmation by mass spectrometry", *Anal. Chim. Acta*, **2005**, *529*, 145-150.
7. A. Townshen, W. Ruengsitagoon, C. Thongpoon, and S. Liawruangrath, "Flow injection chemiluminescence determination of tetracycline", *Anal. Chim. Acta*, **2005**, *541*, 103-109.
8. C. M. C. M. Couto, J. L. F. C. Lima, M. Conceição, B. S. M. Montenegro, and S. Reis, "Tetracycline, oxytetracycline and chlortetracycline determination by flow injection potentiometry", *J. Pharm. Biomed. Anal.*, **1998**, *18*, 527-533.
9. P. Masawat and J. M. Slater, "The determination of tetracycline residues in food using a disposable screen-printed gold electrode (SPGE)", *Sens. Actuators B*, **2007***, 24*, 127-132.
10. R. Karlíček and P. Solich, "Flow-injection spectrophotometric determination of tetracycline antibiotics", *Anal. Chim. Acta*, **1994**, *285*, 9-12.
11. P. Masawat, "Design and fabrication of low-cost home-made all-in-set colorimetric detector with PC-interface embed for the determination of tetracycline antibiotics", *NU Sci. J.,* **2007**, *3*, 215-230.
12. Z. Fang, "Flow Injection Separation and Preconcentration", VCH Publisher, Weinheim, **1993**.
13. D. A. Skoog, D. M. West, and H. Muller, "Fundamentals of Analytical Chemistry", 6th Edn., Harcourt Brace & Company, Florida, **1992**.

Mj. Int. J. Sci. Tech. **2008**, *2*(02), 418-430

14. H. Oka, Y. Ito, and H. Matsumoto, "Chromatographic analysis of tetracycline antibiotics in foods" *J. Chromatogr. A*, **2000**, *882*, 109-133.